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Published in:
Current Opinion in Microbiology

DOI:
10.1016/j.mib.2018.02.004

Publication date:
2018

Available online at www.sciencedirect.com

ScienceDirect

Current Opinion in Microbiology

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In the past decades, numerous instances of phenotypic diversity were observed in clonal microbial populations, particularly, on the gene expression level. Much less is, however, known about phenotypic differences that occur on the level of metabolism. This is likely explained by the fact that experimental tools probing metabolism of single cells are still at an early stage of development. Here, we review recent exciting discoveries that point out different causes for metabolic heterogeneity within clonal microbial populations. These causes range from ecological factors and cell-inherent dynamics in constant environments to molecular noise in gene expression that propagates into metabolism. Furthermore, we provide an overview of current methods to quantify the levels of metabolites and biomass components in single cells.

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Current Opinion in Microbiology 2018, **45**:30–38

This review comes from a themed issue on **Microbial systems biology**

Edited by **Terence Hwa** and **Uwe Sauer**

https://doi.org/10.1016/j.mib.2018.02.004

Introduction

In the last two decades, we have obtained ample evidence that cellular phenotypes are determined not only by the genotype and environment, but can be influenced also by stochastic effects. These stochastic effects arise from Brownian motion of low copy number biomolecules involved in gene expression. A range of studies has shown how the stochasticity in gene expression can lead to these different phenotypes, specifically, in terms of transcript and protein levels [1–3].

By contrast, likely due to the lack of experimental tools to probe metabolism in single cells, still relatively little is known how the molecular noise on the gene expression level propagates into metabolism. Recent evidence, nevertheless, suggests that clonal microbial cells can display significant diversity in their metabolism, with the extreme case of subpopulations having distinctly different activities of metabolic pathways [4–6]. Furthermore, recent discoveries show that even under constant conditions metabolism of microbes is not static, but changes over time, for instance, along the cell division cycle [7••] and with ageing [8,9]. The variability of metabolism within clonal populations can pose challenges in eradicating pathogenic microbes, as individual cells can exhibit different degrees of antibiotic tolerance [5]. In the same way, the presence of metabolically low-performing variants may compromise the efficiency of biotechnological processes in industry [10].

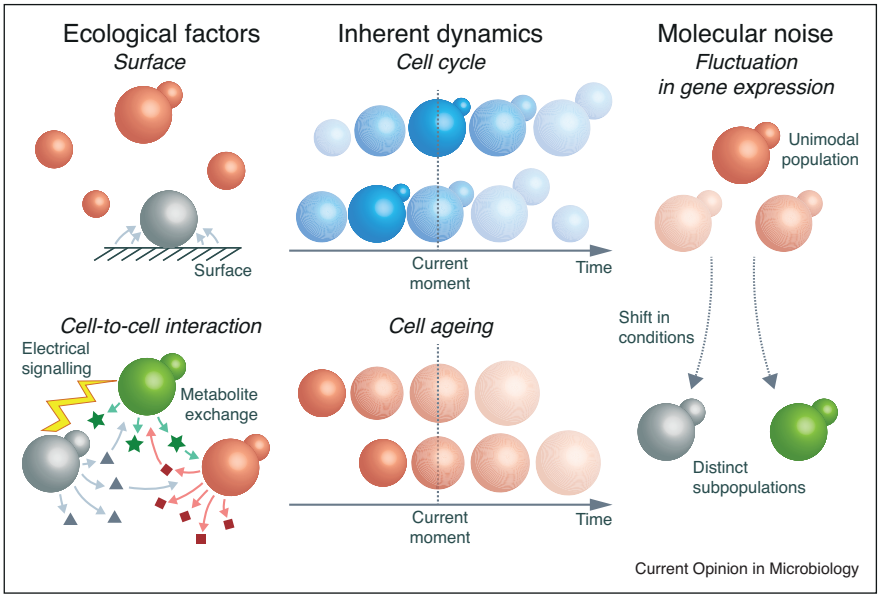
In this review, we gather evidence for metabolic heterogeneity observed in clonal microbial populations. We show recent examples where metabolic heterogeneity arises from ecological factors, cell-inherent dynamics and stochastic effects (Figure 1). Novel experimental tools probing metabolism in single cells are necessary to further uncover metabolic differences within microbial populations and to ultimately understand how such heterogeneity occurs. Thus, we also review the latest developments of respective tools (Table 1) and highlight insights on metabolic differences that have been discovered using these methods.

Metabolic heterogeneity arising from the influence of ecological factors

It is well accepted that environmental factors can cause metabolic heterogeneity. Still, recent work has added new insights into what such ecological factors can be and how they can influence cellular metabolism. Remarkably, it has recently been found that surface immobilization of yeast cells upregulates genes of glycolysis and cell wall biogenesis, as well as accelerates ethanol production and induces glycogen accumulation. Contrary to yeast in planktonic state, the immobilized cells rapidly cease dividing and preserve viability for more than two weeks [11].

Another common ecological factor that can stimulate metabolic diversification is provided by intercellular interaction among the very cells in the population [12]. For instance, within a yeast colony, cells have been found to cooperate by producing different anabolic precursors and eventually exchanging them [13]. Such metabolic specialization of individual cells has been shown to determine their ability to cope with stress [14]. Interestingly, complex behavior can emerge from such intercellular interaction. Cells in the periphery of a *Bacillus subtilis* biofilm are known to starve the interior cells [15]. The latter, in turn, communicate their demand for food using

Figure 1



Sources of metabolic heterogeneity in microbial populations. *Ecological factors*. Environmental factors, such as surface and intercellular communications, can affect individual cells differently and, therefore, stimulate their metabolic divergence. *Inherent dynamics*. In a microbial population, single cells usually lack synchrony in cell division cycle and ageing. Consequently, at a given moment of time, the cells are in various phases of corresponding metabolic changes. *Molecular noise*. Fluctuations in gene expression can generate variability of enzyme levels and metabolic fluxes, including growth rates, among single cells. Together with particular feedback circuits, such heterogeneity may lead to multi-stability of metabolism, for instance, upon shifts in nutrient conditions.

specific metabolites [15] and even electrical signaling [16]. These intercellular interactions have been found to result in periodic cessation of growth of the peripheral cells and nutrient flow into the interior [15]. To efficiently exploit scarce nutrients, even two neighboring biofilms of *B. subtilis* have been found to couple their metabolism via the electrical communication and to undergo anti-phase growth oscillations [17••]. Thus, ecological factors, such as surface attachment and intercellular interactions, which are present even under strictly controlled experimental conditions, may lead to metabolic heterogeneity.

Metabolic heterogeneity arising from cell-inherent dynamics

Experiencing identical environmental factors, two cells can still differ in their metabolism, for instance, due to cell-inherent dynamics. One type of such dynamics is associated with the cell division cycle. Recently, it has been shown in single yeast cells that oscillations in NAD(P)H and ATP levels occur in synchrony with the cell cycle but are autonomous from it [7••]. Transcriptomics and ribosome profiling experiments on synchronized populations suggest that the cells periodically foster biomass production along the cell cycle. Particularly, mitochondrial and ribosomal biogenesis genes are found upregulated in G1, the same happens for lipid biosynthesis genes in G2/M [18•]. Thus, eukaryotic microbes have oscillations in the levels of metabolites, and potentially also in the biosynthesis of biomass components.

This likely extends to prokaryotes as well. For example, in *Caulobacter crescentus* the level of cyclic di-GMP has been found to oscillate and to control the cell cycle [19]. Similarly, recent dynamic single-cell measurements of NAD(P)H levels in *Escherichia coli* have shown that also this metabolite oscillates along the cell cycle [20]. Likewise, the earlier reported non-Gaussian distribution of ATP levels across single cells [21] could possibly have its origin in a cell cycle dynamics. Furthermore, precise measurements of cell size and signals from fluorescent proteins in *E. coli* have suggested that the rate of protein biosynthesis has substantial cell cycle dependence [22]. Thus, there is emerging evidence that prokaryotic microbes also experience metabolic changes during their cell division cycle.

Under constant environments, cellular ageing represents another temporal change of metabolic phenotype and hence is an additional cause for metabolic heterogeneity. Budding yeast ages with every division (replicative ageing), and older cells show increased levels of pyruvate and TCA cycle intermediates and decreased levels of amino acids [8]. Furthermore, it has been found in replicatively ageing yeast that proteins involved in translation become more abundant relative to their transcripts, which

Table 1

Experimental methods to quantify the levels of metabolites and biomass components in single cells.

	Method	Target	Features	Examples
Methods directly exploiting intrinsic properties of metabolites or biomass components	Mass spectrometry (single-cell 'metabolomics')	Absolute amount of primary and secondary metabolites	+ simultaneous measurement of up to 30 compounds + high-throughput − disruptive, that is, non-dynamic	Metabolites of central carbon metabolism [54,45,73], highly abundant lipid species and pigments [46,47]
	Exploiting autofluorescence of metabolites	Concentration of cofactors and pigments	+ non-disruptive, that is, dynamic − mostly non-selective	*Fluorescent microscopy:* NAD(P)H [7••], chlorophyll [54] *FLIM:* decomposed NADH and NADPH [48], fraction of protein-bound to free NAD(P)H [49]
	Raman spectroscopy	Concentration of abundant chemical bonds, biomass components and pigments	+ non-disruptive − mostly non-selective (regarding chemical compounds)	Lipids [50–52], DNA and proteins [51], paramylon and chlorophyll [52], astaxanthin [54], global metabolic profiling [74], carbon–deuterium chemical bond [53•]
Methods assisted by interaction of metabolites with engineered proteins	Transcription factor-based sensors	Concentration of primary and secondary metabolites	+ genetically encoded − non-ratiometric − delayed response (based on the expression of a fluorescent protein)	NADPH [75], NAD^+/NADH [76], malonyl-CoA [55], p-coumaric acid [56], ε-caprolactam, δ-valerolactam and butyrolactam [77]
	Single fluorescent protein-based sensors	Concentration of primary metabolites	+ genetically encoded + immediate response +/− ratiometric or non-ratiometric	*Non-ratiometric:* Trehalose [78], NAD^+/NADH [79] *Ratiometric:* NAD^+/NADH [58], NADH [80], NADPH [57•]
	FRET-based sensors	Concentration of primary metabolites, signaling molecules, metal ions	+ genetically encoded + immediate response + ratiometric − mostly <2-fold response	*Two fluorescent proteins:* Pyruvate [61], trehalose-6-phosphate [81], ATP [82], cAMP [83] *One fluorescent protein:* $NADP^+$ [62]
Methods assisted by interaction of metabolites with engineered RNA	RNA-based sensors expressing fluorescent proteins	Concentration of secondary metabolites (big heterocyclic compounds)	+ genetically encoded + 7–11-fold response − delayed response (current concept) − non-ratiometric	Theophylline, tetracycline and neomycin [66]
	RNA-based sensors with Spinach-like aptamers	Concentration of primary metabolites, signaling molecules (big heterocyclic compounds)	+ genetically encoded + 5–25-fold response + immediate response − require external fluorophore − non-ratiometric	Adenosine, ADP, S-adenosylmethionine, guanine and GTP [63], cyclic di-GMP and cyclic AMP-GMP [68], cyclic di-AMP [69], thiamine 5′-pyrophosphate [67]

suggests that biosynthetic activities change as cells age. Moreover, features of starvation and oxidative stress are also induced at old ages [9]. Surprisingly, yeast cells surviving without nutrients (chronological ageing) are unexpectedly found to reduce the fraction of metabolites containing naturally occurring heavy isotopes [23]. Overall, ageing of the budding yeast is related to changes in metabolism and thus contributes to the metabolic heterogeneity of a population.

Metabolic heterogeneity arising from molecular noise

In addition to ecological factors and cell-inherent temporal behavior, stochasticity in molecular interactions can give rise to heterogeneity among cells. Gene expression involves interactions of molecules present at low copy numbers, which makes the rate of this process prone to fluctuations [1–3]. If flux-limiting metabolic enzymes and transporters are subject to such fluctuations, the noise in gene expression may propagate into the fluxes through metabolic pathways, as indicated in recent studies [24,25]. In fact, it has been shown in *E. coli* that noise in the expression of an individual catabolic enzyme propagates even further through the whole metabolic network finally affecting the growth rate of single cells [26••]. The altered growth rate, in turn, has been found to change the expression of other unrelated genes [26••]. Such apparently growth-rate-related changes of gene expression, however, could also be due to flux-sensing and flux-dependent regulation [27,28]. Overall, these findings indicate that molecular noise in the production of even one enzyme can have global effects on the entire metabolism and expression of genes.

As growth rate represents the ultimate product of each cell's metabolism, we can use its dispersion among cells to assess the metabolic heterogeneity within a population. Here, large scatters of growth rates can be found among single cells in microbial populations [29–31]. Can there be an advantage of such dispersion? Counterintuitively, according to experiments in both bacteria and yeast, having a wider distribution of single cell growth rates does eventually increase the population growth rate [29,30], which suggests that the latter is determined not only by the average cell division time. Besides, at low population growth rates, a bigger dispersion of single cell growth rates makes the population more adaptive to rapid shifts to more favorable conditions [31]. Thus, intercellular metabolic heterogeneity, manifested in growth rate variability, can actually play a beneficial role increasing fitness of the whole population.

Metabolic heterogeneity among cells does not necessarily have a unimodal form. For example, under the same conditions, the microalga *Chlamydomonas reinhardtii* appears in fast and slowly growing subpopulations [32]. The extreme case of metabolic heterogeneity is the occurrence of non-dividing cells in a growing population. In diauxie, after depleting the favorable carbon source, *Lactococcus lactis* forms a subpopulation of dormant cells [4]. Similarly, a shift from glucose to gluconeogenic carbon sources generates bistability in *E. coli*'s central carbon metabolism, resulting in a fraction of non-growing or slowly growing cells [5,33,34•]. Furthermore, starved yeast cells provided with glucose or galactose form a subpopulation that does not manage to balance glycolysis and thus undergoes growth arrest [6]. These findings show that metabolic heterogeneity can be also bimodal. Moreover, it seems that such multiple stable phenotypes tend to emerge from a unimodal population due to a rapid change in the environment.

How can such multi-stability of metabolism emerge within one population? The space of metabolic fluxes could have several attractors, and in a given environment cells would be situated around one of them [35]. A nutrient change could position cells on a different location in the space of metabolic fluxes, where there is comparable influence of two different attractors. If the cells had slightly different metabolic fluxes due to molecular noise, flux-sensing circuits, like *E. coli*'s positive feedback FBP-Cra [28,36] and negative feedback cAMP-Crp [37], would distribute the cells between the two attractors. Thus, building on molecular noise and assisted by nutrient changes, feedback mechanisms could assign cells of one population to different attractors, that is, different metabolic phenotypes.

Cells drawn into an attractor of no or slow growth represent a highly relevant phenomenon as they can turn into so-called persisters, that is, cells that exhibit tolerance towards antibiotics [35]. Recently, the metabolic phenotype of *E. coli*'s persisters has been unraveled, where it was found that their proteome is characterized by a global stress response, and that these cells are metabolically active with upregulated catabolism, very slow buildup of biomass and reduced metabolite pools [34•]. Persisters of the Gram-positive bacterium *S. aureus* have been found to have decreased levels of ATP [38], which may reflect a strong perturbation of their metabolic homeostasis, which was found to trigger persistence in *E. coli* [34•,35]. Interestingly, recent findings indicate that persisters occur not only in bacteria. Cells resistant to harsh environmental conditions, including drug treatment, also appear in yeast [39]. Overall, dormant and, particularly, persister cells are an example of extreme metabolic heterogeneity that can be present in a clonal population of microbes.

Experimental tools to uncover metabolic properties of single cells

To further uncover metabolic heterogeneity in microbial populations and mechanisms generating it, single-cell measurements of metabolic properties are required. However, here we are still confronted with grand challenges.

First, unlike proteins, metabolites cannot be fluorescently tagged. Second, even if the concentration of a metabolite can be measured, in most cases the concentration does not say much about the activity of the corresponding metabolic pathway.

Below, we illustrate recent exciting developments that have been made towards quantifying the levels of metabolites and biomass components in single cells. Measuring fluxes through specific metabolic pathways in single cells is, however, still unattainable. Only the resulting activity of many metabolic pathways, for example, the flux to biomass, that is, the growth rate, can be gauged, even with high precision. Next to determining single-cell growth rates from microscopic time-lapse data [29–31], a recent technology exploits resonant mass sensors to measure the *buoyant* mass and hence the growth rate of single cells in high-throughput manner [40]. Another microresonator set-up has quantified the *total* mass of a single cell and unexpectedly revealed its fluctuations in the second range, which seem to be linked to water transport and ATP synthesis [41•].

Metabolic activity can also be crudely assessed in single cells via the assimilation of externally provided compounds, that is, the accumulation of certain atoms inside the cells. Particularly, nanometer-scale secondary ion mass spectrometry (NanoSIMS) has identified heterogeneity in the activity of CO_2 and N_2 fixation within the population of *Chlorobium phaeobacteroides* incubated with the labelled gases [42]. Furthermore, recent NanoSIMS experiments have shown that *S. aureus* cells incorporate heavy water with markedly different rates [43]. Of note, the cells analyzed in these two studies were extracted from natural habitats, thus the identified metabolic variability could also be due to genetic differences. A recent NanoSIMS study, using a clonal *E. coli* population grown on a mixture of isotope-labelled arabinose and glucose, has disclosed that individual cells assimilate these sugars with different preferences [44•]. Thus, NanoSIMS with the help of labelled nutrients can assess the resulting assimilation flux in single cells and identify metabolic heterogeneity among them.

To measure metabolite levels in single microbial cells, mass spectrometry can be utilized (Table 1). Here, through microarrays for mass spectrometry (MAMS), single or few cells have been isolated in 'wells' with subsequent matrix-assisted laser desorption/ionization (MALDI) of their content. With this technique, 19 metabolites of central carbon metabolism could be identified in tens of single yeast cells. On the basis of such data, two subpopulations with either low or high amounts of fructose-1,6-bisphosphate could be distinguished [45]. Recent experiments employing MAMS have allowed high-throughput screening of thousands of individual *C. reinhardtii* cells with measuring 22 highly abundant lipids and pigments [46]. With another technique, aerosol time-of-flight mass spectrometry (ATOFMS), individual cells of the same alga experiencing four days of nitrogen limitation have been analyzed at the frequency of 50 Hz. It has been found that on the second day the variability among cells in the level of one lipid is 40% bigger than on any other day, suggesting a subpopulation with slower response to nitrogen deprivation [47]. Given the high throughput possibility of these single-cell mass spectrometric techniques, it should now be possible to screen individual cells of entire microbial populations. However, the number of identified metabolites is still rather limited, and their levels are only qualitatively assessed.

Single-cell concentrations of some metabolites can be assessed by exploiting their intrinsic spectroscopic properties, particularly their autofluorescence. In a recent study using the autofluorescence of NAD(P)H, it has been shown that metabolite oscillations exist in single cells of budding yeast [7••]. Assessing NAD(P)H concentration via its autofluorescence has recently become possible even in the much smaller cells of *E. coli*, whose metabolism seems to oscillate as well during the cell division cycle [20].

By means of fluorescence-lifetime imaging microscopy (FLIM), it is even possible to decompose the signals of NADPH and NADH in single-cell measurements [48]. Furthermore, the FLIM-phasor technique could determine the ratio between protein-bound to free NAD(P)H molecules. Recently, it has been shown that single cells of diverse bacteria, including *E. coli*, *B. subtilis* and *Staphylococcus epidermidis*, modulate this ratio in response to environmental conditions. Particularly, increased free NAD(P)H appears with antibiotic treatment [49].

With Raman spectroscopy, utilizing the inelastic scattering of light by chemical bonds, it is also possible to measure a number of biomolecules in single microbial cells. Coherent anti-Stokes Raman spectroscopy (CARS) could quantify neutral lipids in individual yeast cells [50]. With stimulated Raman scattering (SRS) microscopy it is feasible to measure DNA as well as proteins and lipids [51]. SRS, being a fast technique, has recently revealed variability in lipid, paramylon and chlorophyll content in the motile microalga *Euglena gracile* [52]. In labelling experiments with heavy water, single-cell Raman microspectroscopy has been elegantly applied to estimate bacterial viability under antibiotic influence, specifically, by tracking substitution of C–H by C–D bond in macromolecules [53•].

Through application of different single-cell techniques on the very same cell, interesting insights have been obtained. For instance, first, fluorescent and Raman microscopies were used to determine concentrations of the pigments chlorophyll and astaxanthin in single cells of

the algae *Haematococcus pluvialis*. Thereafter, the abundances of 13 primary metabolites were measured in the same cells using MAMS. Here, across the population of cells being in various stages of encystment, it has been found that ATP/ADP ratio anti-correlates with the ratio between astaxanthin and chlorophyll contents [54]. Thus, before disrupting cells for mass spectrometry, they can be studied spectroscopically to maximize the information gain.

If dynamic single-cell measurements of metabolites are necessary, but the metabolites are not fluorescent or cannot be accessed via Raman spectroscopy, then genetically encoded biosensors, selectively reporting metabolite concentrations, are the method of choice. Here, in recent years, three different approaches exploiting interaction of target metabolites with proteins have been explored: transcription factor-based, single fluorescent protein-based and FRET- (i.e. Förster Resonance Energy Transfer) based biosensors (Table 1).

Transcription factors that bind specific metabolites have been engineered into sensors driving the expression of a fluorescent protein and thus providing a proxy for the metabolite concentration. With such transcription factor-based biosensors, populations of *E. coli*, *Corynebacterium glutamicum* and *Saccharomyces cerevisiae* have recently been sorted in search of subpopulations with the highest level of industrially desired metabolites [55,56]. Relying on fluorescent protein expression, these sensors, however, cannot be used for tracking fast changes in metabolite concentration.

Contrarily, another sensor concept, where a metabolite binding domain is fused to a single fluorescent protein and induces changes in its fluorescence, allows reporting transient metabolite concentration. Recently, sensors utilizing this concept have been developed for NADPH [57•] and the ratio of NADH and NAD^+ [58]. Grafting GFP into ammonium transceptors has produced biosensors whose fluorescent response correlates with the transport activity, however, this may represent sensing of extracellular ammonium concentration only rather than the flux itself [59]. The disadvantage of some of such sensors, and also of all transcription factor-based ones, is that their read-out (fluorescence) can be easily confounded by sensor expression levels.

FRET-based sensors are a solution against such confounding effects, because they are intrinsically ratiometric. In most of these sensors, a metabolite binding domain links two fluorescent proteins and affects the efficiency of FRET between them upon metabolite binding. With a FRET-based sensor expressed in *Mycobacterium smegmatis*, it has been found that maintaining high ATP levels during antibiotic exposure correlates with cells' ability to resume growth in normal conditions [60]. A sensor homologous to the previous one has recently disclosed ATP oscillations in single cells of yeast [7••]. Interestingly, a pyruvate FRET-sensor has been used to infer the flux of this metabolite into the mitochondria, with the application of an inhibitor which stops the pyruvate influx into the cell [61]. FRET-based sensors can also rely on one fluorescent protein. Here, a sensor dimerizes upon metabolite binding and causes a decrease in steady-state fluorescence anisotropy, thus reporting the concentration of the substrate. Such sensor has been developed for $NADP^+$ [62]. FRET-sensors are excellent tools offering real-time dynamic read-outs of metabolite concentration. However, the set of protein domains able to bind metabolites and appropriately reorient the fluorescent proteins to enable FRET seems to be limited in nature [63].

Nucleic acid-based sensors might be an alternative, as they could be developed for any metabolite due to efficient *in vitro* selection of aptamers, namely SELEX [64], and versatility of naturally occurring riboswitches [65]. In recent RNA-based sensors, mRNA encoding a fluorescent protein contains an aptamer and a self-cleaving ribozyme so that, upon metabolite binding, the former modulates the latter, eventually controlling translation [66]. In another design, an *in vitro* selected aptamer or riboswitch binds a metabolite and enables another aptamer, specifically Spinach or Spinach2, to activate fluorescence of an externally added chemical. Studies employing such sensors have reported a large cell-to-cell variability in the concentration of S-adenosylmethionine [63] and thiamine 5′-pyrophosphate [67] in cells of an *E. coli* population. RNA-based sensors can lead to 5–25-fold increases in fluorescence upon metabolite binding [63,66–69] which is dramatically higher than rather modest responses of FRET-based sensors. New fluorophores with different spectral properties [70] and recently discovered alternative fluorophore-binding aptamers, like baby Spinach [71] and Broccoli [72], may further fuel development of RNA-based sensors. Such further work is necessary as the currently available RNA-based metabolite sensors can measure only large heterocyclic compounds, and unfortunately not yet the small and negatively-charged metabolites of primary metabolism.

Future avenues

As shown in this review, the cells of clonal microbial populations can exhibit significant metabolic heterogeneity, which in the most extreme case leads to the coexistence of growing and non-growing cells. Many open questions exist, for example, on the source of metabolic oscillations during the cell division cycle and on the origins of ageing-associated changes in metabolism. Further, the mechanisms causing microbial populations to end up in metabolically different phenotypes remain mostly unknown.

Key to unravel these questions will be methods to probe metabolism on the single cell level. Here, significant advances have been made in recent years. Nevertheless, many of the current tools are still at the proof-of-concept level, meaning that thorough *in vivo* validation is necessary before these tools can be routinely applied for actual research. Due to the theoretically unlimited versatility of nucleic acid-based metabolite sensors, we expect that they will become the standard tool to dynamically visualize metabolite levels in single cells, although, admittedly, still many technical problems need to be solved with such sensors.

The ultimate challenge, however, will be to devise methods to sense or visualize the functional output of metabolism, that is, the metabolic fluxes through specific metabolic pathways, in single cells. Measuring metabolic fluxes in single cells, ideally in dynamic fashion, represents an extreme technical challenge, and even on the conceptual level it is not clear how this could be done. One possibility might be to exploit flux-signaling metabolites, whose concentration strictly correlates with the corresponding metabolic flux [27,36], and develop biosensors for these metabolites to obtain dynamic single-cell flux measurements.

Author contribution

VT and MH conceived the study and wrote the manuscript.

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